for this attenuated toxin and completed the present invention.

The objectives mentioned above can be attained by the present invention set forth below:

(1) an adjuvant, comprising an attenuated toxin having a residual toxic activity of less than one-two thousandth (<1/2000) that of the natural toxin corresponding thereto, prepared by attenuating the natural toxin having serine residues, glutamic acid residues, and lysine residues in its amino acid sequence, or by attenuating the subunit thereof;

(2) the adjuvant of (1), wherein the toxin is a mutant comprising the amino acid sequence of the corresponding natural toxin, wherein one or more amino acid residues are substituted, inserted, deleted, and/or added, and having an adjuvant activity;

(3) the adjuvant of (1), wherein the toxin is a natural toxin;

(4) the adjuvant of any of (1) to (3), wherein the toxin is a bacterial toxin;

(5) the adjuvant of (4), wherein the toxin is selected from the group consisting of cholera toxin, pertussis toxin, heat-labile toxin of pathogenic *E. coli*, Staphylococcus α toxin and β toxin, and thermostable hemolytic toxin of *Vibrio parahaemolyticus*;

(6) the adjuvant of (5), wherein the toxin is a natural cholera toxin having a toxic activity of less than one-two thousandth that of the corresponding natural toxin, prepared by attenuating a natural cholera toxin with formalin treatment;

(7) the adjuvant of (6), wherein the toxic activity is less than one-ten thousandth that of the corresponding natural toxin;

(8) a vaccine preparation comprising an adjuvant of any one of (1) to (7) and one or more vaccine antigens;

(9) the vaccine preparation of (8), wherein the vaccine preparation is formulated for intranasal administration;

(10) the vaccine preparation of (8), wherein the vaccine preparation is formulated for oral administration;

(11) the vaccine preparation of (8), wherein the vaccine preparation is formulated for percutaneous administration;

(12) the vaccine preparation of (8), wherein the one or more vaccine antigens comprise antigens from one or more pathogenic

microorganisms selected from the group consisting of influenza virus, rotavirus, measles virus, rubella virus, mumps virus, AIDS virus, *Bordetella pertussis*, diphtheria bacillus, *Helicobacter pylori*, enterohaemorrhagic *Escherichia coli* (EHEC), *Chlamydia*, *Mycoplasma*, Malaria parasite, coccidium protozoa, and schistosome.

The present invention also relates to the use of an attenuated toxin as an adjuvant. The present invention further relates to the use of an attenuated toxin in the production of vaccine preparations.

The toxin composing the adjuvant of this invention is an attenuated toxin that has substantially no toxic activity but still has the activity to enhance immunity. "Having substantially no toxic activity" is defined herein as having toxic activity that is less than one-two thousandth relative to that of the natural toxin. The activity of a representative bacterial toxin, such as cholera toxin, *E. coli* heat-labile toxin, pertussis toxin, diphtheria toxin, or tetanus toxin, can be compared by, for example, using the ADP-ribosyltransferase activity as an index. That is, for example, when the ADP-ribosyltransferase activity is reduced to one-two thousandth, then it means that the toxic activity is reduced to one-two thousandth.

Some technical papers so far reported described that ADP-ribosyltransferase was not detectable in various attenuated toxins. However, it may be improper to evaluate the toxic activity without any quantitative comparison, because the criterion is unclear in such evaluation. Further, it is problematic because it does not allow comparison of results of two papers. In the present invention, a value of one-two thousandth of the natural toxic activity was adopted as an index in order to avoid possible confusion. Even when the toxin is of bacterial origin like cholera toxin or *E. coli* heat-labile toxin, having a strong physiological activity, the high level of attenuation, wherein the toxicity is reduced to one-two thousandth that of the natural toxin, improve, to a great extent, the safety of adjuvants when injected to a human body, while retaining the adjuvant's immuno-stimulating activity.

In addition to the ADP-ribosyltransferase activity, the results obtained from bioassays utilizing physiological responses of animal

cells or experimental animals may also be used as an index of toxic activity, allowing the quantitative comparison.

In any case, it is preferable to verify that the toxicity is reduced to at least one-two thousandth that of the natural toxin by comparing the relative activities using an index that sensitively reflects the activity of toxin. Further, even if a sensitive index is used, it is also desirable in terms of safety to evaluate the toxic activity by the combined use of multiple indexes, each reflecting different mechanisms, because many toxins have pleiotropic physiological activities. Particular toxins and their activities are specified later. The toxic activity of the attenuated toxin of the invention is typically reduced to at least one-two thousandth that of the natural toxin, and is reduced to at least one-ten thousandth in preferred embodiments.

Attenuation of toxins can be attained by conventional methods. For example, chemical treatments and physical treatments can be used as techniques for the attenuation of toxins. The attenuation treatments can result in various structural changes, such as irreversible changes in three-dimensional structure, dissociation of subunits, or fragmentation of peptides. However, the adjuvant activity can be maintained in the toxin by retaining the above-mentioned three types of amino acid residues (e.g., serine, glutamic acid and lysine) in it. With such known methods, the condition where the toxic activity becomes at least one-two thousandth that of the natural toxic activity, as measured by any of the above-mentioned indexes representing the toxic activity, can be determined empirically. Specific conditions of attenuation are described later in detail.

The toxins of the present invention, specific examples of which are indicated later, must retain serine residues, glutamic acid residues and lysine residues contained in the amino acid sequence of the natural toxin. A preferred toxin of the present invention is a natural toxin. A high adjuvant activity is expectable; moreover, the steps required for screening mutants may be avoided by selecting a natural toxin.

In another embodiment, the toxin of the present invention can be a mutant which has the amino acid sequence of the natural toxin,